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## REMARKS/ARGUMENTS

Claims 1, 2, and 6-22 are pending. Favorable reconsideration is respectfully requested.

The present invention relates to a transformed microorganism belonging to enterobacteria and having L-glutamic acid productivity, into which a citrate synthase gene obtained from a coryneform bacterium is introduced.

See Claim 1.

The present invention also relates to a process for producing L-glutamic acid comprising

isolating a coryneform bacterium citrate synthase gene by amplifying the gene with oligonucleotide primers comprising SEQ ID NOS: 1 and 2;

transforming a enterobacteria with said isolated citrate synthase gene;

culturing said enterobacteria in a liquid medium to produce and accumulate the L-glutamic acid; and

collecting the L-glutamic acid produced.

See Claim 11.

The rejection of the claims under 35 U.S.C. §112, first paragraph, for an alleged lack of written description, is respectfully traversed.

As noted above, Claim 1 specifies a citrate synthase gene obtained from a coryneform bacterium. Page 16, line 8 to page 18, bottom describes the coryneform bacteria in great detail. Many specific examples of those bacteria are provided, many of which are available from depositories. At page 18, lines 17-18, the specification provides a reference to a scientific publication which describes the nucleotide sequence of a citrate synthase gene (*glt*A) from a coryneform bacterium. That publication is *Microbiology*, 140, 1817-1828 (1994), a copy of which is submitted herewith. The nucleic acid sequence and the encoded

amino acid sequence are shown on pages 1822-1823 of that publication. Importantly, the specification provides the sequences of two nucleotide primers, i.e., SEQ ID NO: 1 and 2, that can be used to obtain the citrate synthase gene from coryneform bacteria via PCR. See page 18, lines 18-23 of the specification.

All of the coryneform bacteria described in the specification have a citrate synthase gene. Since those bacteria are all corneform, the description of one sequence in the literature, i.e., as shown by the *Microbiology* publication, is sufficient.

The Examiner has criticized the specification for not describing "yet to be discovered polynucleotides encoding citrate synthase" (see page 3 of the Official Action). Of course, the present specification does not describe polynucleotides that have not been discovered yet. That's impossible! In fact, the Federal Circuit agrees, stating in the recent Chiron Corporation v. Genentech, Inc. (70 USPQ2d 1321, Fed. Cir. 2004) decision that

> a patent document cannot enable technology that arises after the date of application. The law does not expect an applicant to disclose knowledge invented or developed after the filing date. Such disclosure would be impossible. [Page 1325-1326.]

In view of the foregoing, the Inventors had possession of the full scope of the invention at time the present application is satisfied. Accordingly, the present application satisfies the written description requirement of 35 U.S.C. §112, first paragraph. Withdrawal of this ground of rejection is respectfully requested.

The rejection of the claims under 35 U.S.C. §112, first paragraph, for an alleged lack of enablement, is respectfully traversed.

The specification of the present application provides a detailed description for producing a transformed microorganism belonging to enterobacteria and having L-glutamic acid productivity, into which a citrate synthase gene derived from a coryneform bacterium is introduced. The level of detail provided is such that one can prepare the microorganism

using routine experimentation. Since routine experimentation is not undue, the claims are enabled.

Pages 6-16 of the specification provide a detailed description for selecting the enterobacteria used in the present invention. Many specific examples of enterobacteria are provided, along with a detailed description of their biological properties. In fact, several of these microorganisms are available from commercial depositories. See, for example, the bottom of page 7 of the specification.

Page 16, line 8 to page 18, bottom describes the coryneform bacteria in great detail. Again, many specific examples of those bacteria are provided, many of which are available from depositories. As discussed above, page 18, lines 17-18 the specification provides a reference to a scientific publication which describes the nucleotide sequence of a citrate synthase gene (*glt*A) from a coryneform bacterium. Importantly, the specification provides the sequences of two nucleotide primers, i.e., SEQ ID NO: 1 and 2, that can be used to obtain the citrate synthase gene from coryneform bacteria via PCR. See page 18, lines 18-23 of the specification.

Beginning at the bottom of page 18, the specification provides explicit guidance for introducing the citrate synthase gene derived from coryneform bacteria into enterobacteria. Specific examples of vectors are given along with a detailed explanation of the transformation procedure. Again, specific citations to the scientific literature are provided at page 20. In addition, detailed guidance for culturing the microorganism is provided at pages 24-25.

At pages 26-30 of the specification, explicit and specific examples of the procedures for preparing the claimed microorganism are provided.

The Examiner has criticized the specification for not describing "yet to be discovered polynucleotides encoding citrate synthase" (see page 3 of the Official Action). Of course, the

present specification does not describe polynucleotides that have not been discovered yet. That's impossible! In fact, the Federal Circuit agrees, stating in the recent Chiron Corporation v. Genentech, Inc. (70 USPQ2d 1321, Fed. Cir. 2004) decision that

> a patent document cannot enable technology that arises after the date of application. The law does not expect an applicant to disclose knowledge invented or developed after the filing date. Such disclosure would be impossible. [Page 1325-1326.]

In view of detailed teaching provided in the specification of the present application, which includes specific citations to the scientific literature and explicit examples, one can obtain the claimed microorganism using routine experimentation. Routine experimentation is not undue experimentation. Therefore, the claims are enabled. Withdrawal of this ground of rejection is respectfully requested.

Applicants submit that the rejection of the claims under 35 U.S.C. §101 is obviated by the amendment submitted above in part and is respectfully traversed in part.

Claim 1 has been amended to recite "transformed," in accordance with the Examiner's suggestion. Claims 6-10, 16-19, and 20-23 depend directly or indirectly from Claim 1.

Claim 11 is directed to a method and is believed to recite the "hand of man" in its current form. Claims 12-15 and 24-27 depend from Claim 11.

In view of the foregoing, withdrawal of this ground of rejection is respectfully requested.

Applicants submit that the present application is in condition for allowance. Early notice to this effect is earnestly solicited.

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# Nucleotide sequence, expression and transcriptional analysis of the *Corynebacterium glutamicum gltA* gene encoding citrate synthase

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**Citrate synthase catalyses the initial reaction of the citric acid cycle and can therefore be considered as the rate-controlling enzyme for the entry of substrates into the cycle. In *Corynebacterium glutamicum*, the specific activity of citrate synthase was found to be independent of the growth substrate and of the growth phase. The enzyme was not affected by NADH or 2-oxoglutarate and was only weakly inhibited by ATP (apparent $K_i$ = 10 mM). These results suggest that in *C. glutamicum* neither the formation nor the activity of citrate synthase is subject to significant regulation. The citrate synthase gene, *gltA*, was isolated, subcloned on plasmid pJC1 and introduced into *C. glutamicum*. Relative to the wild-type the recombinant strains showed six- to eightfold higher specific citrate synthase activity. The nucleotide sequence of a 3007 bp DNA fragment containing the *gltA* gene and its flanking regions was determined. The predicted *gltA* gene product consists of 437 amino acids ($M_r$ 48936) and shows up to 49·7% identity with citrate synthase polypeptides from other organisms. Inactivation of the chromosomal *gltA* gene by gene-directed mutagenesis led to absence of detectable citrate synthase activity and to citrate (or glutamate) auxotrophy, indicating that only one citrate synthase is present in *C. glutamicum*. Transcriptional analysis by Northern (RNA) hybridization and primer extension experiments revealed that the *gltA* gene is monocistronic (1·45 kb mRNA) and that its transcription initiates at two consecutive G residues located 121 and 120 bp upstream of the translational start.**

Keywords: *Corynebacterium glutamicum*, *gltA* gene, citrate synthase

## INTRODUCTION

*Corynebacterium glutamicum* is an aerobic, Gram-positive organism which is widely used in the industrial production of amino acids, e.g. L-glutamate and L-lysine (Liebl, 1991). During recent years, considerable progress has been made in the development of genetic techniques for this organism (Martin, 1989; Schwarzer & Pühler, 1991) and this has allowed the study of the structure, organization, expression and regulation of *C. glutamicum* genes and enzymes. Several genes involved in amino acid biosynthesis, especially in L-lysine, L-threonine and L-isoleucine synthesis, have been characterized (reviewed in Eikmanns *et al.*, 1993). Recently, some genes involved in the central metabolism of *C. glutamicum* have also been analysed, e.g. that for the anaplerotic enzyme phosphoenolpyruvate carboxylase (Eikmanns *et al.*, 1989; O'Regan *et al.*, 1989) and those for the glycolytic enzymes fructose-1,6-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase and triosephosphate isomerase (von der Osten *et al.*, 1989; Eikmanns, 1992). Reyes *et al.* (1991) also reported the cloning of the citrate synthase gene from *C. melassecola*, a close relative of *C. glutamicum*; however, no structural data of the gene were presented.

**Abbreviation:** WT, wild-type.

The EMBL/GenBank/DDBJ accession number for the sequence reported in this paper is X66112.

Citrate synthase (EC 4.1.3.7) catalyses a crucial step at the entry of the citric acid cycle, i.e. the condensation of acetyl-CoA and oxaloacetate to form citrate and CoA. The key position of this enzyme within central metabolism has given rise to much interest in its structural, kinetic, regulatory and molecular characteristics and, therefore, it has been studied in great detail from a variety of different organisms (reviewed by Weitzman, 1981; Wiegand & Remington, 1986; Kay & Weitzman, 1987). All citrate synthases investigated so far are multimers of identical subunits with $M_r$ values of 40000–50000. Gram-positive bacteria and eukaryotic organisms possess dimeric citrate synthases ($M_r \sim 100000$) which are inhibited by ATP, whereas Gram-negative bacteria have in general hexameric forms ($M_r \sim 250000$) which are allosterically inhibited by NADH and, in facultative anaerobes, by 2-oxoglutarate. However, the subunits of both citrate synthase types are approximately the same size and in some regions display identity in their amino acid sequences (Kay & Weitzman, 1987; Sutherland *et al.*, 1990; Schendel *et al.*, 1992).

The citrate synthase of *C. glutamicum* ssp. *flavum* (formerly *Brevibacterium flavum*) has been partially purified and shown to have features typical of the Gram-positive-type enzyme: an $M_r$ of about 92000, sensitivity to ATP and insensitivity towards NADH and 2-oxoglutarate (Shiio *et al.*, 1977). The same authors reported that a classically obtained mutant of *C. glutamicum* ssp. *flavum* with reduced citrate synthase activity was able to produce significant amounts of aspartate and lysine (Shiio *et al.*, 1982). This and the key position of the enzyme in the carbon flow from carbohydrates to the citric acid cycle, and thus also to the amino acids derived therefrom, suggest that citrate synthase may be an important target in the genetic construction of defined amino-acid-producing *C. glutamicum* strains. We describe here the citrate synthase of *C. glutamicum* with respect to its regulation, the isolation of the citrate synthase gene (*gltA*), its nucleotide sequence, the homologous and heterologous expression and its transcriptional organization.

## METHODS

**Bacteria, plasmids and media.** The bacterial strains, plasmids, their relevant characteristics and their source or reference are given in Table 1. The minimal medium used for *C. glutamicum* has been described previously (Eikmanns *et al.*, 1991b). M9 medium (Sambrook *et al.*, 1989) was used as minimal medium for *Escherichia coli* and LB medium (Sambrook *et al.*, 1989) was used as complex medium for both organisms. For the growth of *E. coli* W620 on minimal medium, L-glutamate was added at 15 mM. When appropriate, ampicillin (50 μg $ml^{-1}$) or kanamycin (50 μg $ml^{-1}$) was added to the medium. *E. coli* was grown aerobically at 37 °C, *C. glutamicum* at 30 °C.

**DNA preparation, transformation and conjugation.** For the isolation of chromosomal DNA from *C. glutamicum*, the cells were grown in 5 ml LB medium to the late exponential growth phase, washed in 10 mM Tris/1 mM EDTA (TE) buffer, pH 7·6, and incubated in 1 ml TE containing 15 mg lysozyme for 180 min at 37 °C. Then 3 ml TE, pH 8·2, containing 400 mM NaCl, 220 μl 10% (w/v) SDS and 3 mg proteinase K was added and the mixture was incubated for 5 h at 50 °C. After adding 1 ml saturated NaCl (~ 6 M), shaking slightly for 2 min and centrifugation for 15 min at 13000 *g*, the DNA in the supernatant was ethanol-precipitated and resuspended in 200 μl TE. Plasmids from *E. coli* were isolated as described by Birnboim (1983), and those from *C. glutamicum* were isolated by the same method with prior incubation (1 h, 37 °C) of the cells with lysozyme (15 mg $ml^{-1}$). *E. coli* was transformed by the $CaCl_2$ method (Sambrook *et al.*, 1989). *C. glutamicum* was transformed by electroporation as described by Liebl *et al.* (1989). Conjugation between *E. coli* S17-1 and *C. glutamicum* was performed as described by Schäfer *et al.* (1990) and transconjugants were selected on LB agar plates containing kanamycin (25 μg $ml^{-1}$) and nalidixic acid (50 μg $ml^{-1}$).

**DNA manipulations.** Restriction enzymes, T4 DNA ligase, Klenow polymerase, calf intestine phosphatase, proteinase K, DNase I (RNase-free) and RNase A were obtained from Boehringer Mannheim and used as specified by the manufacturer.

For DNA hybridization, 10 μg *SalI–Hin*dIII-restricted chromosomal DNA from *C. glutamicum* wild-type (WT) was size-fractionated on 0·8% agarose gels and transferred onto a nylon membrane Nytran 13 (Schleicher und Schüll) by vacuum-supported diffusion using the VacuGene system from Pharmacia. A 1·85 kb *SalI–XhoI* fragment from plasmid pKK3-1 was labelled with digoxigenin-dUTP and used as a probe. Blotting, labelling, hybridization, washing, detection and size determination were performed using the non-radidactive 'DNA Labeling and Detection Kit' and the instructions from Boehringer Mannheim.

For sequencing, the *SalI–Hin*dIII fragment was cloned in either orientation into plasmid pUC18 and progressive unidirectional deletions of the inserted DNA were made using the Erase-a-Base system from Promega. Appropriate subclones were sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the T7 sequencing kit, fluorescence-labelled primers and the A.L.F. sequencer from Pharmacia. The sequence data were compiled and analysed on an IBM PC using the sequence analysis program Microgenie from Beckmann.

**RNA analysis.** Total RNA from *C. glutamicum* was isolated as previously described by Börmann *et al.* (1992) except that the phenol extraction temperature was 65 °C. The RNA concentration was determined by measuring the $A_{280}$.

For Northern (RNA) hybridization, a *gltA*-antisense RNA probe was prepared as follows. The 1·15 kb *BclI–Nru*I fragment (see Fig. 1) was isolated from plasmid pKK3-1 and ligated into *Bam*HI–*Sma*I-restricted plasmid pGEM-3Z. After linearization with *Hin*dIII, digoxigenin-dUTP-labelled RNA was synthesized using T7 RNA polymerase and the 'RNA Labeling Kit (SP6/T7)' from Boehringer Mannheim. For hybridization, 10 μg total RNA from *C. glutamicum* WT was incubated with 1 U DNase I (10 min at 37 °C), mixed with loading dye (Sambrook *et al.*, 1989), heated for 5 min at 95 °C, cooled in ice-water and loaded on an agarose gel containing 17% (v/v) formaldehyde. The separated RNA was transferred onto a nylon membrane as described for DNA blotting (see above). Hybridization to the *gltA* antisense RNA probe (at 44 °C, in the presence of 50%, v/v, formamide), washing and detection were performed using the 'Nucleic Acid Detection Kit' and the instructions from Boehringer Mannheim. The size marker was the 0·24–9·5 kb RNA ladder from Gibco-BRL.

For the ribonuclease protection assay, the 0·5 kb *BglI–BclI* fragment (see Fig. 1) was isolated from plasmid pKK3-1 (the *BglI* site being blunt-ended by treatment with Klenow polymerase before restriction with *BclI*) and ligated into

***Table 1.*** Bacterial strains and plasmids

| Strain/plasmid | Relevant characteristics* | Source/reference |
|---|---|---|
| **Strains** | | |
| *C. glutamicum* WT | WT strain ATCC 13032 | American Type Culture Collection |
| *E. coli* W620† | *supE44 thi-1 pyrD36 gltA6 galK30 rpsL129 glnV44* | Reissig & Wollmann (1963) |
| *E. coli* DH5 | *supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1* | Hanahan (1985) |
| *E. coli* S17-1 | Mobilizing donor strain | Simon *et al.* (1983) |
| **Plasmids** | | |
| pHC79 gene library | *C. glutamicum* chromosomal DNA cloned in cosmid pHC79 | Börmann *et al.* (1992) |
| cos-pKK3 | pHC79 containing a 31 kb chromosomal *Sau*3A fragment from *C. glutamicum* | This work |
| pBR322 | $Ap^R$ $Tet^R$ | Bolivar *et al.* (1977) |
| pUC18 | $Ap^R$ | Vieira & Messing (1982) |
| pKK3-1 | pBR322 containing a 7·4 kb *Bam*HI fragment from cos-pKK3 | This work |
| pJC1 | *E. coli–C. glutamicum* shuttle vector, $Ap^R$ $Km^R$ | Cremer *et al.* (1990) |
| pJC-gltA7A and B | pJC1 containing a 7·4 kb *Bam*HI fragment from cos-pkk3 | This work |
| pJC-gltA3A and B | pJC1 containing a 3 kb *Sal*I–*Hin*dIII fragment from pJC-gltA7A | This work |
| pEKEx1 | Expression vector carrying $lacI^q$ and the *tac* promoter | Eikmanns *et al.* (1991a) |
| pEKEx2 | pEKEx1 containing the pUC18 multiple cloning site | This work |
| pEKEx2-gltA | pEKEx2 containing the 3 kb *Sal*I–*Hin*dIII *gltA* fragment | This work |
| pSUP301 | Mobilizable vector, *oriT*, $Km^R$ | Simon *et al.* (1983) |
| pSUP-gltA$_{int}$ | pSUP301 containing a 0·54 kb internal *gltA* fragment | This work |
| pGEM-3Z | Transcription vector carrying the T7 and SP6 promoters, $Ap^R$ | Promega Corp. |

* $Ap^R$, ampicillin resistance; $Km^R$, kanamycin resistance; $Tet^R$, tetracycline resistance.

† This strain was kindly provided by Barbara Bachmann, *E. coli* Genetic Stock Center, USA.

*Sma*I–*Bam*HI-restricted plasmid pGEM-3Z. After linearization of the resulting vector with *Sac*I, a radiolabelled transcript was synthesized using 50 µCi (1·85 MBq) [$^{35}$S]CTPαS (1000 Ci mmol$^{-1}$; 37000 GBq mmol$^{-1}$) (Amersham) and the SP6/T7 Transcription Kit from Boehringer Mannheim. Hybridization to the RNA from *C glutamicum* and the protection assay was performed as described by Börmann *et al.* (1992).

For the primer extension experiments, 30 µg vacuum-dried RNA was dissolved in 100 µl 40 mM PIPES buffer, pH 6·4, containing 1 mM EDTA, 0·4 M NaCl and 80% (v/v) formamide. After adding 10 pmoles primer (5′-TTATCAGTAGC-CACGATATC-3′, complementary to the sequence from position 844 to 863 in Fig. 2 and synthesized using the 'Gene Assembler Plus' and appropriate chemicals from Pharmacia), denaturation at 95 °C (10 min) and hybridization at 52 °C (8 h), the mixture was ethanol-precipitated (−20 °C), washed once with 70% (v/v) ethanol and vacuum-dried. The pellet was dissolved in 20 µl 50 mM Tris/HCl buffer (pH 7·6) containing 60 mM KCl, 10 mM $MgCl_2$, 250 µM dCTP, 250 µM dGTP, 250 µM dTTP, 2·5 µM dATP and 40 U RNasin (Promega). Then 30 µCi (1·1 MBq) [$^{35}$S]dATPαS and 5 U avian myeloblastosis virus (AMV) reverse transcriptase (Promega) were added and after incubation for 2 h at 42 °C the reaction was stopped by adding 1 µl 0·5 M EDTA (pH 8). Subsequently, the RNA was removed by incubation with RNase A (addition of 1 µl of a 1 µg ml$^{-1}$ solution) for 30 min at 37 °C. After ethanol precipitation and drying under vacuum, the primer extension product was dissolved in 3 µl TE plus 3 µl formamide buffer (80% formamide, 10 mM EDTA, 0·1% xylene cyanole, 0·1% bromphenol blue) and 2 µl of this mixture was loaded onto a 6% (w/v) polyacrylamide sequencing gel. For exact localization of the transcriptional start site, sequencing reactions using plasmid pKK3-1 and the same oligonucleotide used for the primer extensions were co-electrophoresed.

**Enzyme assay.** To determine the citrate synthase activity, cells were grown in 60 ml medium in baffled 500 ml Erlenmeyer flasks to the exponential growth phase, washed twice in 25 ml Tris buffer (50 mM, pH 7·5) containing 200 mM sodium glutamate and resuspended in 2 ml of the same buffer. Cells were disrupted by sonication as described (Eikmanns, 1992). After centrifugation for 30 min at 13000 *g*, the supernatant was used for the assays. Citrate synthase was assayed spectrophotometrically at 412 nm and 30 °C as described by Srere (1969) except that the assay contained 200 mM sodium glutamate. The protein concentration was determined by the method of Bradford (1976) using egg albumin as the standard.

## RESULTS AND DISCUSSION

### Citrate synthase activity in *C. glutamicum*

Citrate synthase activity was determined in *C. glutamicum* WT after growth on LB medium with and without glucose (2%, w/v) and on minimal medium containing glucose (4%, w/v), acetate (2%, w/v), lactate (2%, w/v) or glutamate (2%, w/v) as carbon source. The specific activity of the enzyme was in all cases between 0·5 and 0·8 µmol min$^{-1}$ (mg protein)$^{-1}$ and it was thus independent of the growth substrate. When the cells were harvested at the early, mid or late exponential phase or at the stationary growth phase, the specific citrate synthase activity was also identical. These results indicate that *gltA* expression is constitutive and not regulated at the

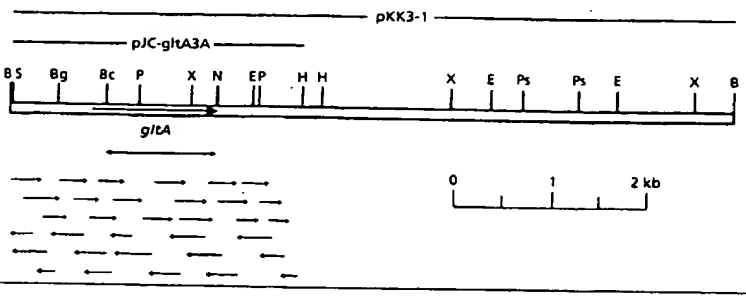


***Fig. 1.*** Restriction map of the *C. glutamicum* chromosomal 7·4 kb DNA fragment and sequencing strategy for the 3·0 kb *SalI–HindIII* fragment containing the *gltA* gene. The bars above the map indicate those regions which are present in plasmids pKK3-1 and pJC-gltA3A (and pJC-gltA3B), respectively. Thin arrows indicate the direction and the extent of each nucleotide sequence determined. The heavy arrow represents the predicted *gltA* coding region; the double-headed arrow indicates the *BclI–NruI* fragment used for the preparation of the Northern (RNA) hybridization probe. B, *BamHI*; Bc, *BclI*; Bg, *BglI*; E, *EcoRI*; H, *HindIII*; N, *NruI*; P, *PvuII*; Ps, *PstI*; S, *SalI*; X, *XhoI*.

transcriptional or translational level. In contrast, the expression of the *E. coli* and *Bacillus subtilis* citrate synthase genes was reported to be repressed by the presence of glucose in the growth medium (Gray *et al.*, 1966; Flechtner & Hanson, 1969; Wilde & Guest, 1986).

Characterization of the citrate synthase activity in cell-free extracts of *C. glutamicum* WT revealed apparent $K_m$ values of 1·5 μM for oxaloacetate and 51 μM for acetyl-CoA. The enzyme was weakly inhibited by ATP (apparent $K_i = 10$ mM) and *cis*-aconitate (apparent $K_i = 15$ mM). No effect was observed with NADH, NAD, NADPH, NADP, ADP, AMP or with any intermediate of the citric acid cycle, except *cis*-aconitate. These data are in accordance with previously described data obtained with the partially purified citrate synthase of *C. glutamicum* ssp. *flavum* (Shiio *et al.*, 1977) and suggest that the citrate synthases of both *C. glutamicum* WT and ssp. *flavum* are identical.

## Isolation of the *C. glutamicum gltA* gene

The *C. glutamicum gltA* gene encoding citrate synthase was isolated by heterologous complementation of the *E. coli gltA* mutant W620 using a *C. glutamicum* WT cosmid gene library based on vector pHC79. Pooled recombinant cosmids were transformed into *E. coli* W620 and approximately 4000 transformants were obtained. They were screened for complementation of the *gltA* marker by replica-plating onto glucose minimal medium. Seven transformants showed a $gltA^+$ phenotype, i.e. they grew in the absence of glutamate. Restriction analysis of the respective recombinant cosmids revealed a common 7·4 kb *Bam*HI fragment. This fragment was isolated from cosmid cos-pKK3 and ligated into the *Bam*HI-restricted plasmid pBR322, resulting in plasmid pKK3-1. Transformation of this plasmid into *E. coli* W620 resulted in a $gltA^+$ phenotype of all transformants, suggesting that the 7·4 kb *Bam*HI fragment in fact contains the *C. glutamicum gltA* gene. The restriction map of the 7·4 kb *Bam*HI fragment is shown in Fig. 1.

In order to localize the *gltA*-complementing region more precisely within the 7·4 kb fragment, several smaller fragments were ligated in plasmid pUC18 and tested for complementation ability. A plasmid containing the 3·0 kb *SalI–HindIII* fragment (see Fig. 1) was able to complement the *gltA* mutation, suggesting that the *C. glutamicum gltA* gene is located within this fragment.

Southern hybridization was performed to confirm that the cloned *SalI–HindIII* fragment originated from *C. glutamicum*. Chromosomal DNA from *C. glutamicum* WT was digested with *SalI* and *HindIII*, size-fractionated and transferred onto a nylon membrane. A digoxigenin-dUTP-labelled 1·85 kb *SalI–XhoI* fragment isolated from pKK3-1 was used as a probe. This fragment hybridized specifically to a chromosomal DNA fragment of 3·0 kb (not shown). This result confirmed that the cloned *gltA*-complementing fragment originates from *C. glutamicum* and that it corresponds to a fragment within the genome with no detectable structural alterations.

## Expression of the cloned *gltA* gene

For homologous expression of the *gltA* gene in *C. glutamicum*, both the 7·4 kb *Bam*HI and the 3·0 kb *SalI–HindIII* fragments were ligated in both orientations into the *C. glutamicum–E. coli* shuttle vector pJC1 resulting in plasmids pJC-gltA7A, pJC-gltA7B, pJC-gltA3A and pJC-gltA3B, respectively. These vectors were introduced into *C. glutamicum* WT and the citrate synthase activities of the transformants and of the parental strain were determined (Table 2). The recombinant *C. glutamicum* strains showed six- to eightfold higher specific activity compared with the host strain. These results prove that the isolated fragments contain the functional *gltA* gene and indicate the presence of a promoter in front of this gene. It is noteworthy that the *gltA*-overexpressing *C. glutamicum* strains showed slower growth on all media tested (e.g. on LB medium a doubling time of 120 min instead of 80 min) indicating a slight impairment of the cells by the enhanced level of citrate synthase.

Citrate synthase activity was also determined in *E. coli* W620 carrying cos-pKK and pKK3-1, respectively. As shown in Table 2, distinct activity was present in these

***Table 2.*** Specific activities of citrate synthase in cell-free extracts of *C. glutamicum* WT, *E. coli* W620, *E. coli* DH5, and recombinant derivatives thereof

Cells were grown, harvested, disrupted and their enzyme activities were measured as described in Methods. The values are means ± standard deviation.

| Strain | Citrate synthase activity [μmol $min^{-1}$ $(mg\ protein)^{-1}$]* |
|---|---|
| *C. glutamicum* WT | 0·7 ± 0·1 (11) |
| *C. glutamicum* WT(pJC-gltA7A) | 6·3 ± 0·4 (4) |
| *C. glutamicum* WT(pJC-gltA7B) | 5·2 ± 0·6 (2) |
| *C. glutamicum* WT(pJC-gltA3A) | 4·7 ± 0·1 (4) |
| *C. glutamicum* WT(pJC-gltA3B) | 5·6 ± 0·2 (2) |
| *C. glutamicum* WT*gltA*::pSUP-gltA$_{int}$ | < 0·01 |
| *E. coli* W620 | < 0·01 |
| *E. coli* W620(cos-pKK3) | 0·02 ± 0·00 (2) |
| *E. coli* W620(pKK3-1) | 0·14 ± 0·01 (2) |
| *E. coli* DH5 | 0·30 ± 0·05 (4) |
| *E. coli* DH5(pEKEx2-gltA) | 0·56 ± 0·03 (2) |
| *E. coli* DH5(pEKEx2-gltA)† | 9·6 ± 0·7 (2) |

* Values in parentheses are the total number of experiments.

† Isopropyl-β-D-thiogalactopyranoside at a final concentration of 0·5 mM was added to the growth medium 1 h before harvesting the cells.

strains. However, the relatively low specific activity of citrate synthase indicated that the corynebacterial *gltA* promoter only works poorly in the heterologous *E. coli* host. In order to test this hypothesis, we ligated the *gltA*-containing fragment in both orientations into the expression vector pEKEx2 which provides the IPTG-inducible *tac* promoter. The recombinant plasmid pEKEx2-gltA, in which the *tac* promoter reads from the *Sal*I site into the fragment, conferred a strikingly high specific citrate synthase activity to *E. coli* DH5 when induced with IPTG (Table 2). This result confirms that the promoter of the *C. glutamicum gltA* gene does not function well in *E. coli* and shows that the orientation of the *gltA* gene is in the direction from the *Sal*I to the *Hin*dIII site.

It is well-known that *C. glutamicum* secretes large amounts of glutamate under certain conditions (Liebl, 1991). To test the effect of increased citrate synthase activity on glutamate secretion, standard glutamate fermentations (Hoischen & Krämer, 1989) were performed with *C. glutamicum* WT and WT(pJC-gltA3A). In these experiments identical glutamate secretion rates of about 17 μmol $min^{-1}$ (g dry weight)$^{-1}$ were found for both strains. Thus, the capacity of *C. glutamicum* to secrete glutamate cannot be enhanced by simply elevating the citrate synthase enzyme level. This finding, together with the observation that citrate synthase in *C. glutamicum* is highly active, constitutively formed and subject to only weak regulation (see above), indicates that (i) the chromosomally encoded citrate synthase activity is sufficient for glutamate production by this organism and (ii) a factor other than citrate synthase limits the rate of carbon flow into the citric acid cycle.

## DNA sequence analysis of the *gltA* gene

The DNA sequence of the 3007 bp *Sal*I–*Hin*dIII fragment was determined from both strands by the dideoxy chain-termination method using the strategy depicted in Fig. 1. The nucleotide sequence obtained and the deduced amino acid sequence of the *C. glutamicum* citrate synthase are shown in Fig. 2.

One major open reading frame (ORF) was found extending from nucleotide 805 to 2142 of the *Sal*I–*Hin*dIII fragment. No other ORF of significant size or codon bias was found in either orientation. The predicted translational initiation site at nucleotide 832 is the first ATG within the ORF. Alternatively, the in-frame GTG codon at position 850 might be the translational start site. All other potential translational start codons are located downstream of regions within the amino acid sequence which show significant identity to *gltA* gene products from one or several other organisms (see below). These sites are therefore unlikely candidates. Assuming initiation at $ATG_{832}$, the *C. glutamicum gltA* gene product consists of 437 amino acids with an $M_r$ of 48936, which is in good agreement with the $M_r$ values of purified citrate synthase monomers from other organisms ($M_r$ about 48000) (Weitzman, 1981). However, the monomers from *Bacillus megaterium* and *Bacillus* sp. C4 have been reported to be significantly smaller, with $M_r$ values of 40300 and 42000, respectively (Robinson *et al.*, 1983; Schendel *et al.*, 1992).

Downstream of the *gltA* gene, at positions 2172–2220 in Fig. 2, a palindromic structure followed by a stretch of T residues was found, which is a typical feature of rho-independent transcription terminators in *E. coli* (Rosenberg & Court, 1979). Calculated according to the rules of Tinoco *et al.* (1973), the mRNA stem-loop predicted from this sequence has a $\Delta G$ (25 °C) of −24·4 kcal $mol^{-1}$ (102·1 kJ $mol^{-1}$). This result indicates transcriptional termination downstream of the *gltA* gene.

The codon usage in the *C. glutamicum gltA* gene is given in Table 3. Twelve codons are heavily favoured, i.e. CTG (L), ATC (I), TCC (S), CCA (P), ACC (T), TAC (Y), CAC (H), CAG (Q), AAC (N), AAG (K), GAG (E) and CGC (R). All but one (CCA for P) have a C or a G in the wobble position reflecting the high G+C content (57%) of *C. glutamicum* (Liebl, 1991). Fourteen codons, with two exceptions having an A or T in the wobble position, are not used at all. This shows the highly biased codon usage in the *gltA* gene. When comparing this codon usage profile with that in other *C. glutamicum* genes it is obvious that the codon preference in the *gltA* gene perfectly matches the preference pattern recently compiled for highly expressed genes of *C. glutamicum* (Eikmanns, 1992). However, although this codon preference differs signi-

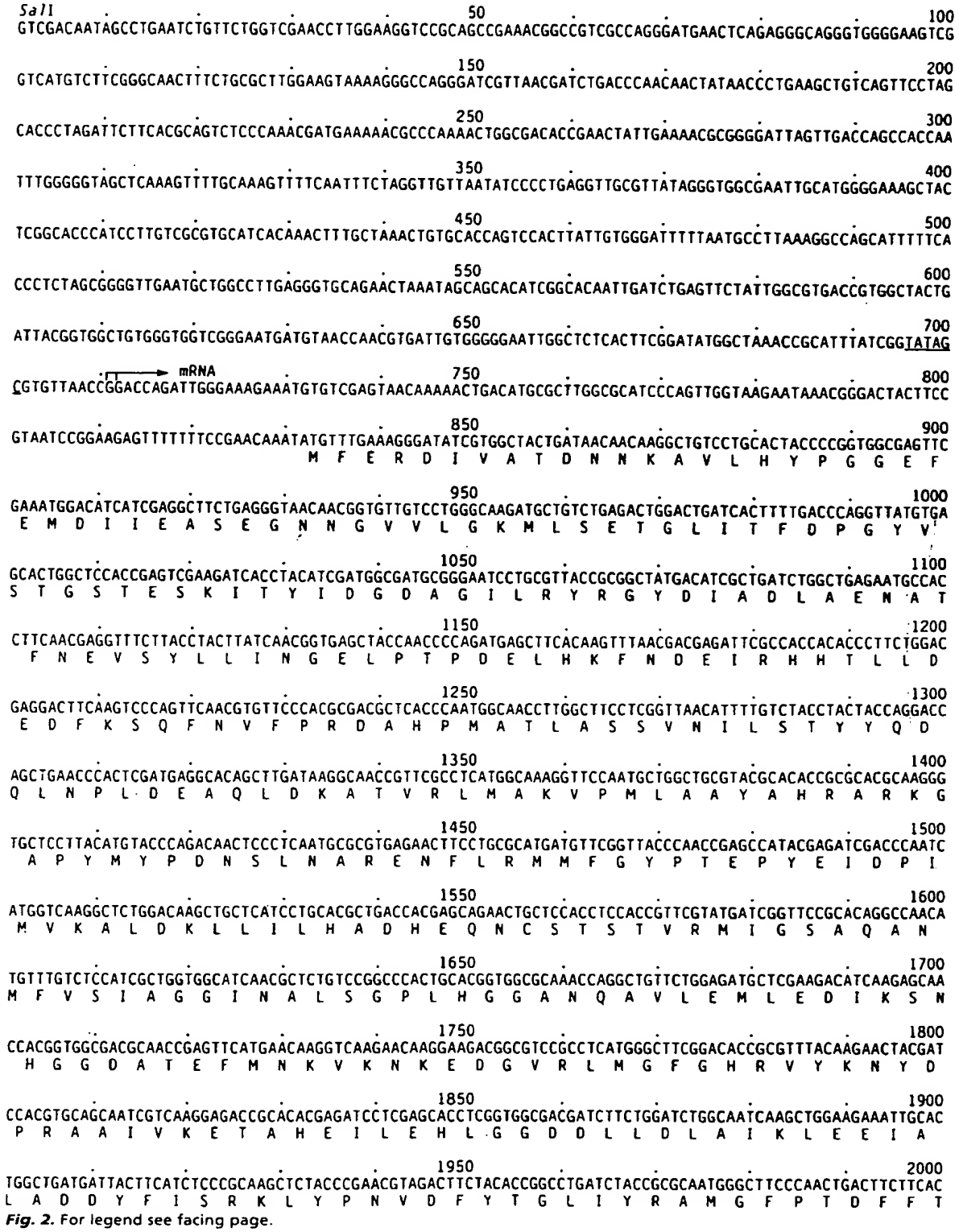


***Fig. 2.*** For legend see facing page.

```
                                                2050                                                2100
CGTATTGTTCGCAATCGGTCGTCTGCCAGGATGGATCGCTCACTACCGCGAGCAGCTCGGTGCAGCAGGCAACAAGATCAACCGCCCACGCCAGGTCTAC
  V  L  F  A  I  G  R  L  P  G  W  I  A  H  Y  R  E  Q  L  G  A  A  G  N  K  I  N  R  P  R  Q  V  Y

                                                2150                                                2200
ACCGGCAACGAATCCCGCAAGTTGGTTCCTCGCGAGGAGCGCTAAATTTAGCGGATGATTCTCGTTCAACTTCGGCCGAAGCCACTTCGTCTGTCATAAT
  T  G  N  E  S  R  K  L  V  P  R  E  E  R  *

                                                2250                                                2300
GACAGGGATGGTTTCGGCCGTTTTTGCATGAAACCAAAAAATACGATTTTCAAGGAGCATGTACAGCACATGGAAAAGCCACAGATTGAGCTACCGGTCG

                                                2350                                                2400
GTCCAGCACCGGAAGATCTCGTAATCTCTGACATCATCGTTGGCGAAGGAGCAGAAGCCCGCCCAGGTGGAGAAGTTGAGGTCCACTATGTGGGCGTTGA

                                                2450                                                2500
CTTTGAAACCGGCGAGGAGTTTGACTCTTCCTGGGATCGTGGACAGACCAGCCAGTTCCCACTCAACGGCCTCATTGCAGGTTGGCAAGAGGGAATTCCA

                                                2550                                                2600
GGCATGAAGGTCGGCGGACGTCGTCAGCTGACCATTCCGCCAGAGGCTGCTTACGGCCCTGAGGGTTCCGGCCACCCACTGTCTGGCCGTACCCTGGTGT

                                                2650                                                2700
TCATCATCGATTTGATCAGCGCATAATTTTCTTTACTGCGCTAAACGCTCAAATCGTGTGAAGCGACTGTCGCGTCCCGCCCTCTCCGGATTGTTATCCA

                                                2750                                                2800
ATTCGGAGAGGGCGTTGCTGATTGTGCCGAGAATTTCTTCAACAAAGTGCTCGGTTTCGGCGACGATCCCGTCGATAAGCCCTTGGCTTAAAAGTGCGTG

                                                2850                                                2900
CGCCTGCACGCCTTGTCGCTCTATGATTTCCGCGGCGTGGTTGGTGTCGCGGAAGAGGATGGCCGAGGCGCCCTCTGGTGGCAATGCGGACAGCCACGCG

                                                2950                                                3000
TTTTCGGCCGCGTAGACCAGATCGGCGGGCAGCATGGCCAGCGCGCCACCGCCAACGCCCTGACCAATAATGACCGAAACGGTGGGGAGGGGAGCGTCGA

 HindIII
TAAGCTT
```

***Fig. 2.*** Nucleotide sequence of the 3007 bp *SalI–HindIII* fragment and the deduced citrate synthase amino acid sequence. The transcriptional initiation site (↦), the putative −10 region (——), the stop codon (*) and a potential transcription terminator (inverted arrows) are shown.

ficantly from that of *E. coli* (Grosjean & Fiers, 1982), translation of the *gltA* gene in this host was quite effective as evidenced by the high specific citrate synthase activity in *E. coli* DH5(pEKEx2-glt) (Table 2).

### Inactivation of the chromosomal *gltA* gene

The chromosomal *gltA* gene of *C. glutamicum* WT was disrupted by gene-directed mutagenesis (Schwarzer & Pühler, 1991). For this purpose, a 0·54 kb internal *PvuII–XhoI* fragment was inserted into the mobilizable *E. coli* vector pSUP301, which is nonreplicative in *C. glutamicum*. The resulting plasmid, pSUP-gltA$_{int}$, was introduced into *C. glutamicum* WT via conjugation from *E. coli* S17-1 and 70 transconjugants were obtained by selection on medium containing kanamycin. Kanamycin resistance indicated integration of pSUP-gltA$_{int}$ into the chromosomal *gltA* gene via recombination between the plasmid-borne *gltA* fragment and the respective region on the *C. glutamicum* chromosome. DNA from six transconjugants was analysed by agarose gel electrophoresis, confirming the absence of autonomous plasmids.

The transconjugant *C. glutamicum* WT*gltA*::pSUP-gltA$_{int}$ was then tested for its ability to grow on different media and for citrate synthase activity. Whereas the growth of the cells on complex medium was only slightly slower than that of the parental strain (doubling times of 100 and 80 min, respectively) the transconjugant showed no growth on solid and liquid minimal medium unless supplemented with citrate (5 mM) or glutamate (5 mM). In accordance with this finding, *C. glutamicum* WT*gltA*::pSUP-gltA$_{int}$ was devoid of any detectable citrate synthase activity (Table 2). These results verify that the *gltA* gene in the transconjugants is inactivated.

The consequences of *gltA* disruption in *C. glutamicum* WT (citrate/glutamate auxotrophy and lack of citrate synthase activity) and the fact that we observed only one signal on hybridization to the *gltA* probe (see above) strongly suggest that *C. glutamicum* WT only possesses one citrate synthase. In contrast, several species of *Pseudomonas* were reported to possess two forms of citrate synthase (Mitchell & Weitzman, 1986). In *E. coli* (in a revertant of a citrate-synthase-deficient strain), Patton *et al.* (1993) also recently found a citrate synthase which shows altered kinetic, regulatory and structural properties when compared to the hitherto known enzyme of this organism. In this respect it is noteworthy that, in contrast to *E. coli* and many other organisms, up to now no isoenzymes have been found in *C. glutamicum*.

***Table 3.*** Codon usage in the *C. glutamicum gltA* gene

Values in parentheses represent percentages of the codons used for a given amino acid.

| Amino acid | Codon | Total no. of codon occurrences | Amino acid | Codon | Total no. of codon occurrences | Amino acid | Codon | Total no. of codon occurrences |
|---|---|---|---|---|---|---|---|---|
| F | UUU | 4 (78·9) | P | CCU | 2 (10·0) | N | AAU | 2 (7·7) |
| | UUC | 15 (21·1) | | CCC | 1 (5·0) | | AAC | 24 (92·3) |
| | | | | CCA | 16 (80·0) | | | |
| L | UUA | 0 (0) | | CCG | 1 (5·0) | K | AAA | 0 (0) |
| | UUG | 4 (9·3) | | | | | AAG | 20 (100) |
| | CUU | 5 (11·6) | T | ACU | 5 (23·8) | | | |
| | CUC | 10 (23·3) | | ACC | 16 (76·2) | D | GAU | 13 (41·9) |
| | CUA | 2 (4·6) | | ACA | 0 (0) | | GAC | 18 (58·1) |
| | CUG | 22 (51·2) | | ACG | 0 (0) | | | |
| | | | | | | E | GAA | 7 (22·6) |
| I | AUU | 3 (11·1) | A | GCU | 16 (41·0) | | GAG | 24 (77·4) |
| | AUC | 24 (88·9) | | GCC | 2 (5·1) | | | |
| | AUA | 0 (0) | | GCA | 18 (46·2) | C | UGU | 0 (0) |
| | | | | GCG | 3 (7·7) | | UGC | 1 (100) |
| M | AUG | 16 (100) | | | | | | |
| | | | Y | UAU | 2 (90·5) | W | UGG | 1 (100) |
| V | GUU | 9 (40·9) | | UAC | 19 (9·5) | | | |
| | GUC | 8 (36·4) | | | | R | CGU | 5 (21·7) |
| | GUA | 2 (9·1) | End | UAA | 1 (100) | | CGC | 17 (73·9) |
| | GUG | 3 (13·6) | | UAG | 0 (0) | | CGA | 0 (0) |
| | | | | UGA | 0 (0) | | CGG | 0 (0) |
| S | UCU | 4 (21·1) | | | | | AGA | 0 (0) |
| | UCC | 11 (57·9) | H | CAU | 0 (0) | | AGG | 1 (4·3) |
| | UCA | 0 (0) | | CAC | 14 (100) | | | |
| | UCG | 2 (10·5) | | | | G | GGU | 14 (41·2) |
| | AGU | 0 (0) | Q | CAA | 0 (0) | | GGC | 16 (47·1) |
| | AGC | 2 (10·5) | | CAG | 9 (100) | | GGA | 4 (11·7) |
| | | | | | | | GGG | 0 (0) |

## Comparison of deduced citrate synthase amino acid sequence from *C. glutamicum* with those of other organisms

The deduced amino acid sequence of the *C. glutamicum gltA* gene product was aligned with the citrate synthase sequences of other organisms (Fig. 3). Examples of Gram-negative prokaryotes with hexameric (*E. coli* and *Pseudomonas aeruginosa*) and dimeric (*Rickettsia prowazakii*) enzymes, of a Gram-positive prokaryote (*Bacillus* sp. C4) and of a eukaryotic (pig) were chosen. To maximize similarity several gaps were introduced into the sequences. Surprisingly, the *C. glutamicum* citrate synthase showed the highest degree of identity (48–50 %) to the respective enzymes from Gram-negative organisms (Fig. 3). This was substantiated by comparison with citrate synthases from two further Gram-negative organisms, *Coxiella burnetii* (Heinzen *et al.*, 1991) and *Acinetobacter anitratum* (Donald & Duckworth, 1987), which showed 45·8 and 49·7 % identity, respectively. In contrast, the *C. glutamicum* citrate synthase shared only 27 % identity with that from the Gram-positive *Bacillus* and 33 % with that of pig. The identity to the archaeobacterial enzyme from *Thermoplasma acidophilum* (Sutherland *et al.*, 1990) and the yeast enzyme (Suissa *et al.*, 1984) was also only 33 and 29 %, respectively. The high identity of the *C. glutamicum* citrate synthase with that of the Gram-negative organisms was unexpected since (i) other catabolic *C. glutamicum* enzymes, e.g. the glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase or triosephosphate isomerase, have been shown to have a much higher degree of identity to the respective enzymes from bacilli (up to 62 %) and also to those of several eukaryotes (up to 52 %) (Eikmanns, 1992) and (ii) the regulatory properties of the *C. glutamicum* enzyme suggested greater similarity to the Gram-positive and eukaryotic counterparts.

The alignments of the citrate synthase amino acid sequences showed approximately seven highly conserved regions probably involved in catalytic function. These regions contain all residues (except $R_{164}$ and $R_{46}$, which have no counterpart in any bacterial citrate synthase) shown to participate in the catalytic mechanism of the pig enzyme (Remington *et al.*, 1982; Wiegand & Remington,

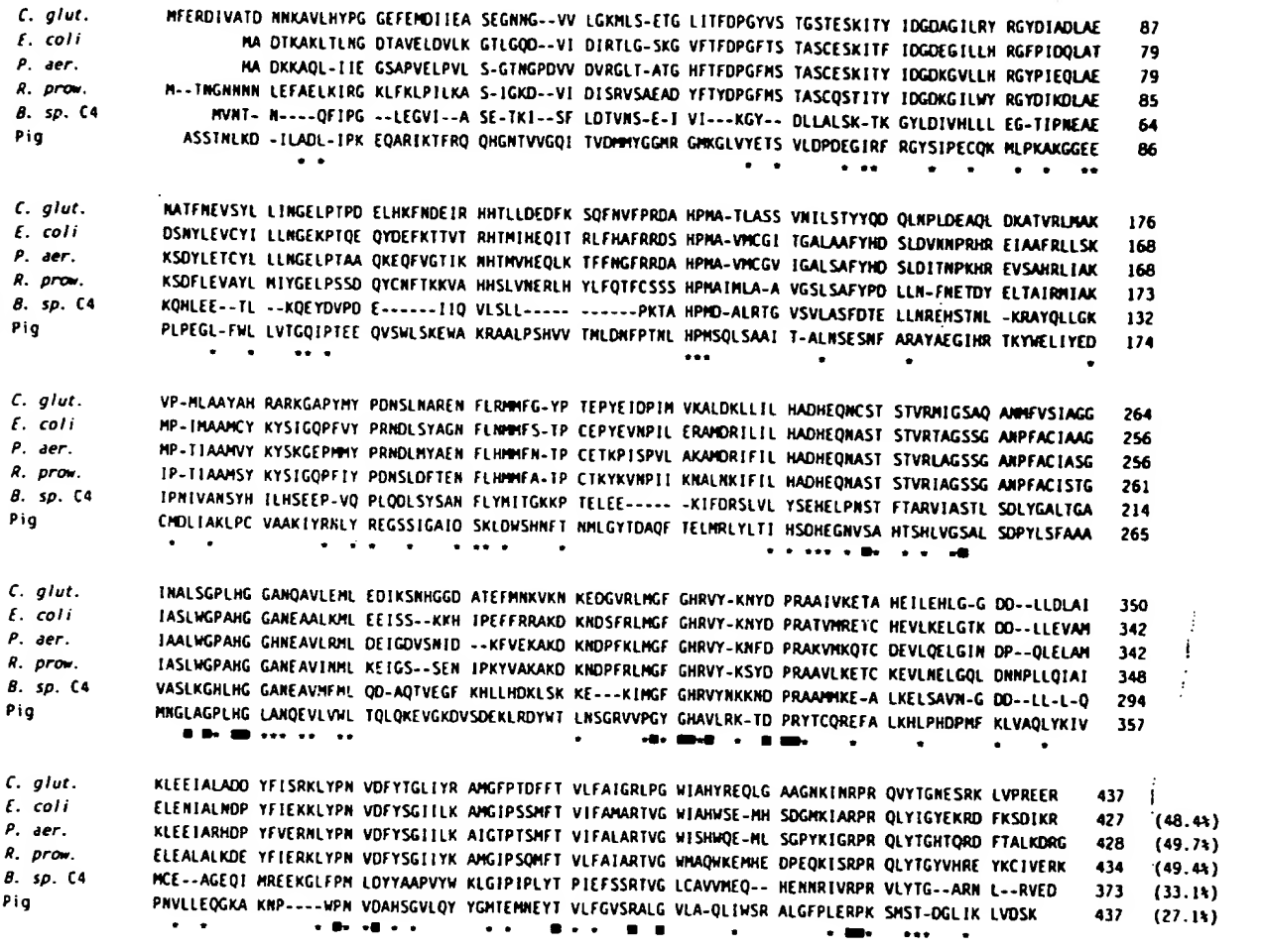

*Fig. 3.* Comparison of the predicted amino acid sequence of the *C. glutamicum* (*C. glut.*) citrate synthase with sequences of citrate synthases from *E. coli* (Ner *et al.*, 1983; Bhayana & Duckworth, 1984), *P. aeruginosa* (*P. aer.*) (Donald *et al.*, 1989), *R. prowazakii* (*R. prow.*) (Wood *et al.*, 1987), *Bacillus* sp. C4 (*B.* sp. C4) (Schendel *et al.*, 1992) and pig (Bloxham *et al.*, 1982). The percentages at the end of the sequences indicate degrees of identity to the *C. glutamicum* citrate synthase. Asterisks mark identical amino acid residues in five out of six citrate synthases tested; solid black squares indicate identical amino acids in all six citrate synthases shown here and, additionally, in the enzymes from *C. burnetii* (Heinzen *et al.*, 1991), *A. anitratum* (Donald & Duckworth, 1987), *T. acidophilum* (Sutherland *et al.*, 1990) and yeast (Suissa *et al.*, 1984).

1986; Alter *et al.*, 1990) and/or of the *E. coli* enzyme (Man *et al.*, 1991), i.e. $H_{235}$, $H_{238}$, $H_{273}$, $H_{316}$, $K_{320}$, $R_{325}$, $D_{372}$, $F_{393}$, $R_{397}$ and $R_{418}$ in the *C. glutamicum* sequence. The $C_{206}$ residue of the *E. coli* citrate synthase shown to be involved in the allosteric inhibition by NADH (Donald *et al.*, 1991) is replaced by T in the *C. glutamicum* and *Bacillus* sp. C4 sequence. This is in accordance with the fact that the citrate synthase of both organisms is insensitive to NADH (Schendel *et al.*, 1992; this work). Interestingly, the alignment revealed some further residues ($S_{243}$, $S_{252}$ and $T_{390}$) which are conserved in all known citrate synthases (including those not shown in Fig. 3) and are not in close proximity to the functionally important residues mentioned above. It is tempting to speculate that they are also critical to the structural, catalytic and/or regulatory properties of citrate synthase.

### Transcriptional analysis of the *gltA* gene

To analyse the size of the *gltA* transcript, Northern (RNA) hybridization experiments were performed. For this purpose, total RNA from *C. glutamicum* WT was isolated, size-fractionated, transferred onto a nylon membrane and hybridized to a *gltA*-specific digoxigenin-UTP-labelled RNA probe. This antisense RNA probe was synthesized by *in vitro* transcription of the 1·15 kb *Nru*I–*Bcl*I fragment (anticlockwise in Fig. 1) cloned downstream of the T7 promoter of vector pGEM-3Z.

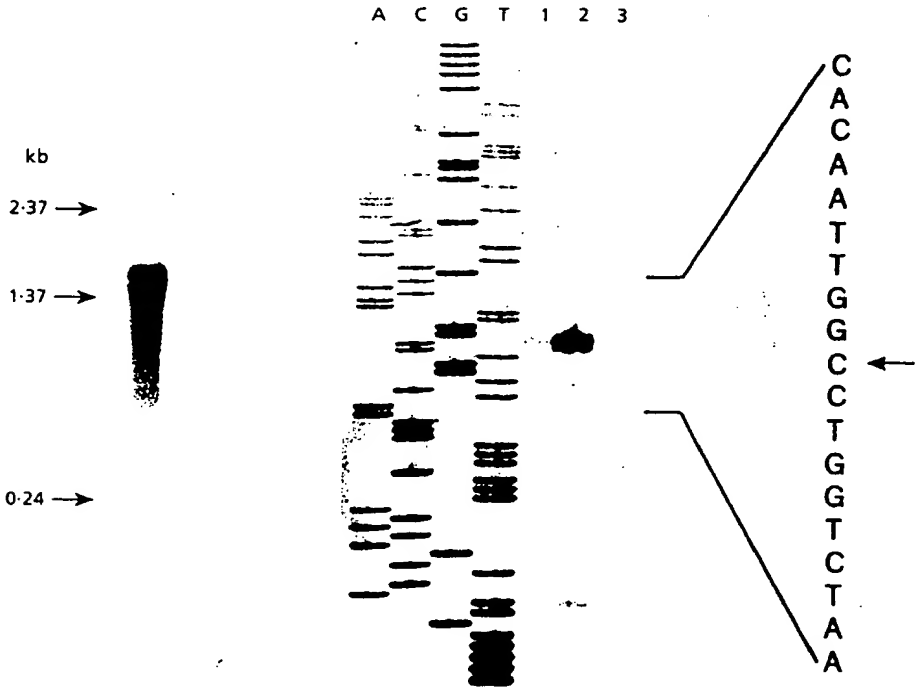


Fig. 4




Fig. 5

***Fig. 4.*** Northern hybridization analysis of the *C. glutamicum gltA* gene. Total RNA from *C. glutamicum* WT was size-fractionated and probed with a digoxigenin-labelled *gltA*-specific antisense RNA probe. RNA standards are shown on the left.

***Fig. 5.*** Primer extension analysis of the transcriptional start site in front of the *gltA* gene. The primer extension products from *C. glutamicum* WT and from the *gltA* overexpresser *C. glutamicum* WT(pJC-gltA3A) are shown in lanes 1 and 2, respectively. A control reaction with total RNA from *C. glutamicum* WT without primer is shown in lane 3. Lanes A, C, G and T represent the products of sequencing reactions using the same primer used for the primer extension and plasmid pKK3-1. The relevant DNA sequence is shown on the right. Note that the sequence represents the coding strand and is thus complementary to that shown in Fig. 2.

The hybridization revealed a signal at 1·45 kb (Fig. 4) indicating that the *C. glutamicum gltA* gene is monocistronic. Thus, the situation is similar to that in *E. coli*, in which the *gltA* gene was also shown to be monocistronic (Wilde & Guest, 1986).

In order to identify the transcriptional start site in front of *gltA*, an RNAse protection assay was performed with 15 μg total RNA isolated from *C. glutamicum* WT and [$^{35}$S]CTPαS-labelled antisense RNA derived from the 0·5 kb *BglI–BclI* fragment at the 5′ end of *gltA* (Fig. 1). This probe was synthesized with SP6 RNA polymerase after cloning of the fragment into pGEM-3Z. The signal obtained was in the size range of 265 bp and corresponded approximately to the region at nucleotides 711–715 in Fig. 2. Since the signal did not allow precise assignment to a specific nucleotide, primer extension experiments with AMV reverse transcriptase and [$^{35}$S]dATPαS were performed. Using an oligonucleotide primer covering the codons 5–11 (nucleotides 863–844 in Fig. 2) and 30 μg total RNA from *C. glutamicum* WT or from *C. glutamicum* WT(pJC-gltA3A) signals were obtained which correspond to the $G_{711}$ residue in Fig. 2 (Fig. 5, lanes 1 and 2, respectively). With the RNA of the *gltA*-overexpressing strain we additionally observed a second less pronounced signal one nucleotide downstream ($G_{712}$ in Fig. 2) and some faint bands which we consider to be negligible (Fig. 4, lane 2). These results show that transcription of the *C. glutamicum gltA* gene starts 121 and 120 nucleotides upstream of the presumed *gltA* translational start. The distance from this transcriptional start site to the assumed terminator structure downstream of the *gltA* gene is 1·5 kb, which is in good agreement with the transcript size determined by Northern blot hybridization (see above).

Recent alignment of the DNA regions upstream of seven experimentally determined transcriptional start sites from *C. glutamicum* genes (Schwinde *et al.*, 1993) revealed in five

cases a 6 bp motif with similarity to typical '−10' regions (TATAAT) of *E. coli* $\sigma^{70}$ (Hawley & McClure, 1983) or *B. subtilis* $\sigma^{43}$ (Moran *et al.*, 1982) promoter consensus sequences. A similar motif (TATAGC) was also found upstream of the *gltA* transcriptional start (Fig. 2). As in the case of several of the corynebacterial promoter regions, no sequence motif with similarity to the '−35' consensus sequence (TTGACA) was found at a proper distance from the *gltA* transcriptional start. The lack of a typical '−35' consensus sequence might be one reason for the weak expression of the *C. glutamicum gltA* gene from plasmids cos-pKK and pKK3-1 in *E. coli*. This, together with the fact that some other *C. glutamicum* genes were not or only very weakly expressed in *E. coli*, e.g. *fda* and *thrC* (von der Osten *et al.*, 1989; Eikmanns *et al.*, 1991b), corroborates the hypothesis that an RNA polymerase containing a sigma factor differing from $\sigma^{70}$ or $\sigma^{43}$ is involved in the transcription of at least some *C. glutamicum* genes.

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## NOTE ADDED IN PROOF

After we had submitted this paper, J. Kalinowski (University of Bielefeld) brought our attention to a further small ORF within the DNA sequence shown in Fig. 2. The ORF starts downstream of the *gltA* gene with an ATG at nucleotide 2270 and stops at nucleotide 2626. The predicted gene product consists of 118 amino acids and showed 50·8% identity to the FK-506-binding protein (a peptidyl-prolyl *cis-trans* isomerase) from *Streptomyces chrysomallus* (Pahl & Keller, 1992).

## REFERENCES

**Alter, G. M., Casazza, J. P., Zhi, W., Nemeth, P., Srere, P. A. & Evans, C. T. (1990).** Mutation of essential catalytic residues in pig citrate synthase. *Biochemistry* **29**, 7557–7563.

**Bhayana, V. & Duckworth, H. W. (1984).** Amino acid sequence of *Escherichia coli* citrate synthase. *Biochemistry* **23**, 2900–2905.

**Birnboim, H. C. (1983).** A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods Enzymol* **100**, 243–255.

**Bloxham, D. P., Parmelee, D. C., Kumar, S., Walsh, K. A. & Titani, K. (1982).** Complete amino acid sequence of porcine heart citrate synthase. *Biochemistry* **21**, 2028–2036.

**Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heynecker, H. L., Boyer, H. W., Cross, J. H. & Falkow, S. (1977).** Construction and characterization of new cloning vehicles. *Gene* **2**, 95–113.

**Börmann, E. R., Eikmanns, B. J. & Sahm, H. (1992).** Molecular analysis of the *Corynebacterium glutamicum gdh* gene encoding glutamate dehydrogenase. *Mol Microbiol* **6**, 317–326.

**Bradford, M. (1976).** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.

**Cremer, J., Eggeling, L. & Sahm, H. (1990).** Cloning the dapA dapB cluster of the lysine-secreting bacterium *Corynebacterium glutamicum*. *Mol & Gen Genet* **220**, 478–480.

**Donald, L. J. & Duckworth, H. W. (1987).** Expression and base sequence of the citrate synthase gene of *Acinetobacter anitratum*. *Biochem Cell Biol* **65**, 930–938.

**Donald, L. J., Molgat, G. F. & Duckworth, H. W. (1989).** Cloning, sequencing, and expression of the gene for NADH-sensitive citrate synthase of *Pseudomonas aeruginosa*. *J Bacteriol* **171**, 5542–5550.

**Donald, L. J., Crane, B. R., Anderson, D. H. & Duckworth, H. W. (1991).** The role of cysteine 206 in allosteric inhibition of *Escherichia coli* citrate synthase. *J Biol Chem* **266**, 20709–20713.

**Eikmanns, B. J. (1992).** Identification, sequence analysis, and expression of a *Corynebacterium glutamicum* gene cluster encoding the three glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, and triosephosphate isomerase. *J Bacteriol* **174**, 6076–6086.

**Eikmanns, B. J., Follettie, M. T., Griot, M. U. & Sinskey, A. J. (1989).** The phosphoenolpyruvate carboxylase gene of *Corynebacterium glutamicum*: molecular cloning, nucleotide sequence, and expression. *Mol & Gen Genet* **218**, 330–339.

**Eikmanns, B. J., Kleinertz, E., Liebl, W. & Sahm, H. (1991a).** A family of *Corynebacterium glutamicum/Escherichia coli* shuttle vectors for cloning, controlled gene expression, and promoter probing. *Gene* **102**, 93–98.

**Eikmanns, B. J., Metzger, M., Reinscheid, D. J. & Sahm, H. (1991b).** Amplification of three threonine biosynthesis genes in *Corynebacterium glutamicum* and its influence on carbon flux in different strains. *Appl Microbiol Biotechnol* **34**, 617–622.

**Eikmanns, B. J., Eggeling, L. & Sahm, H. (1993).** Molecular aspects of lysine, threonine and isoleucine biosynthesis in *Corynebacterium glutamicum*. *Antonie Leeuwenhoek*, **64**, 145–163.

**Flechtner, V. R. & Hanson, R. S. (1969).** Coarse and fine control of citrate synthase from *Bacillus subtilis*. *Biochim Biophys Acta* **184**, 252–262.

**Gray, C. T., Wimpenny, J. W. T. & Mossman, M. R. (1966).** Regulation of metabolism in facultative bacteria. II. Effects of aerobiosis, anaerobiosis and nutrition on the formation of Krebs cycle enzymes in *Escherichia coli*. *Biochim Biophys Acta* **117**, 33–41.

**Grosjean, H. & Fiers, W. (1982).** Preferential codon usage in procaryotic genes: the optimal codon-anticodon interaction energy and the selective codon usage in efficiently expressed genes. *Gene* **18**, 199–209.

**Hanahan, D. (1985).** Techniques for transformation of *E. coli*. In *DNA Cloning, A Practical Approach*, vol. I, pp. 109–135. Edited by D. M. Glover. Oxford: IRL Press.

**Hawley, D. K. & McClure, W. R. (1983).** Compilation and analysis of promoter DNA sequences. *Nucleic Acids Res* **11**, 2237–2255.

**Heinzen, R. A., Frazier, M. E. & Mallavia, L. P. (1991).** Sequence and linkage analysis of the *Coxiella burnetii* citrate synthase-encoding gene. *Gene* **109**, 63–69.

**Hoischen, C. & Krämer, R. (1989).** Evidence of an efflux carrier system involved in the secretion of glutamate by *Corynebacterium glutamicum*. *Arch Microbiol* **151**, 342–347.

**Kay, J. & Weitzman, P. D. J. (1987).** Krebs' citric acid cycle – half a century and still turning. *Biochem Soc Symp* **54**, 1–198.

**Liebl, W. (1991).** The genus *Corynebacterium* – nonmedical. In *The Prokaryotes*, vol. II, pp. 1157–1171. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer Verlag.

**Liebl, W., Bayerl, A., Schein, B., Stillner, U. & Schleifer, K. H.**

**(1989).** High efficiency electroporation of intact *Corynebacterium glutamicum* cells. *FEMS Microbiol Lett* **65**, 299–304.

**Man, W.-J., Li, Y., O'Connor, D. & Wilton, D. C. (1991).** Conversion of citrate synthase into citryl-CoA lyase as a result of mutation of the active-site aspartic acid residue to glutamic acid. *Biochem J* **280**, 521–526.

**Martin, J. F. (1989).** Molecular genetics of amino acid-producing corynebacteria. In *Society for General Microbiology Symposium 44*, pp. 25–59. Edited by S. Baumberg, I. Hunter & M. Rhodes. Cambridge: Cambridge University Press.

**Mitchell, C. G. & Weitzman, P. D. J. (1986).** Molecular size diversity of citrate synthases from *Pseudomonas* species. *J Gen Microbiol* **132**, 737–742.

**Moran, C. P., Lang, N., LeGrice, S. F. J., Lee, G., Stephens, M., Sonenshein, A. L., Pero, J. & Losick, R. (1982).** Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. *Mol & Gen Genet* **186**, 339–346.

**Ner, S. S., Bhayana, V., Bell, A. W., Giles, I. G., Duckworth, H. W. & Bloxham, D. P. (1983).** Complete sequence of the *glt.A* gene encoding citrate synthase in *Escherichia coli*. *Biochemistry* **22**, 5243–5249.

**O'Regan, M., Thierbach, G., Bachmann, B., Villeval, D., Lepage, P., Viret, J.-F. & Lemoine, Y. (1989).** Cloning and nucleotide sequence of the phosphoenolpyruvate carboxylase-coding gene of *Corynebacterium glutamicum* ATCC 13032. *Gene* **77**, 237–251.

**Pahl, A. & Keller, U. (1992).** Fk-50b-binding proteins from Streptomycetes producing immunosuppressive macrolactones of the Fk-50b type. *J Bacteriol* **174**, 5888–5894.

**Patton, A. J., Hough, P. W., Towner, P. & Danson, M. J. (1993).** Does *Escherichia coli* possess a second citrate synthase gene? *Eur J Biochem* **214**, 75–81.

**Reissig, L. J. & Wollmann, E. L. (1963).** Transduction des marqueurs galactose par les bactériophages tempérés 82 et 434 d'*Escherichia coli*. *Ann Inst Pasteur* **105**, 774–779.

**Remington, S., Wiegand, G. & Huber, R. (1982).** Crystallographic refinement and atomic models of two different forms of citrate synthase at 2·7 and 1·7 Å resolution. *J Mol Biol* **158**, 111–152.

**Reyes, O., Guyonvarch, A., Bonamy, C., Salti, V., David, F. & Leblon, G. (1991).** 'Integron'-bearing vectors: a method suitable for stable chromosomal integration in highly restrictive corynebacteria. *Gene* **107**, 61–68.

**Robinson, M. S., Danson, M. J. & Weitzman, P. D. J. (1983).** Citrate synthase from a Gram-positive bacterium. *Biochem J* **213**, 53–59.

**Rosenberg, M. & Court, D. (1979).** Regulatory sequences involved in the promotion and termination of RNA transcription. *Annu Rev Genet* **13**, 319–353.

**Sambrook, J., Fritsch, E. F. & Maniatis, J. (1989).** *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

**Sanger, F., Nicklen, S. & Coulson, A. R. (1977).** DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.

**Schäfer, A., Kalinowski, J., Simon, R., Seep-Feldhaus, A. H. & Pühler, A. (1990).** High frequency conjugal plasmid transfer from gram-negative *Escherichia coli* to various gram-positive coryneform bacteria. *J Bacteriol* **172**, 1663–1666.

**Schendel, F. J., August, P. R., Anderson, C. R., Hanson, R. S. & Flickinger, M. C. (1992).** Cloning and nucleotide sequence of the gene coding for citrate synthase from a thermotolerant *Bacillus* sp. *Appl Environ Microbiol* **58**, 335–345.

**Schwarzer, A. & Pühler, A. (1991).** Manipulation of *Corynebacterium glutamicum* by gene disruption and replacement. *Bio/Technology* **9**, 84–87.

**Schwinde, J. W., Thum-Schmitz, N., Eikmanns, B. J. & Sahm, H. (1993).** Transcriptional analysis of the *gap-pgk-tpi-ppc* gene cluster of *Corynebacterium glutamicum*. *J Bacteriol* **175**, 3905–3908.

**Shiio, I., Ozaki, H. & Ujigawa, K. (1977).** Regulation of citrate synthase in *Brevibacterium flavum*, a glutamate-producing bacterium. *J Biochem* **82**, 395–405.

**Shiio, I., Ozaki, H. & Ujigawa-Takeda, K. (1982).** Production of aspartic acid and lysine by citrate synthase mutants of *Brevibacterium flavum*. *Agric Biol Chem* **46**, 101–107.

**Simon, R., Priefer, U. & Pühler, A. (1983).** A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. *Bio/Technology* **1**, 784–791.

**Srere, P. A. (1969).** Citrate synthase. *Methods Enzymol* **13**, 3–11.

**Suissa, M., Suda, K. & Schatz, G. (1984).** Isolation of the nuclear yeast genes for citrate synthase and fifteen other mitochondrial proteins by a new screening method. *EMBO J* **3**, 1773–1781.

**Sutherland, K. J., Henneke, C. M., Towner, P., Hough, D. W. & Danson, M. J. (1990).** Citrate synthase from the thermophilic archaebacterium *Thermoplasma acidophilum*. *Eur J Biochem* **194**, 839–844.

**Tinoko, I., Borer, P. N., Dengler, B., Levine, M. D., Uhlenbeck, O. C., Crothers, D. M. & Gralla, J. (1973).** Improved estimation of secondary structure in ribonucleic acid. *Nat New Biol* **246**, 40–41.

**Vieira, J. & Messing, J. (1982).** The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**, 259–268.

**Von der Osten, C. H., Barbas, C. F., Wong, C. H. & Sinskey, A. J. (1989).** Molecular cloning, nucleotide sequence and fine structural analysis of the *Corynebacterium glutamicum fda* gene: structural comparison of *C. glutamicum* fructose-1,6-bisphosphate aldolase to class I and class II aldolases. *Mol Microbiol* **3**, 1625–1637.

**Weitzman, P. D. (1981).** Unity and diversity in some bacterial citric acid-cycle enzymes. *Adv Microb Physiol* **22**, 185–243.

**Wiegand, G. & Remington, S. J. (1986).** Citrate synthase: structure, control, and mechanism. *Annu Rev Biophys Biophys Chem* **15**, 97–117.

**Wilde, R. J. & Guest, J. R. (1986).** Transcript analysis of the citrate synthase and succinate dehydrogenase genes of *Escherichia coli*. *J Gen Microbiol* **132**, 3239–3251.

**Wood, D. O., Williamson, L. R., Winkler, H. H. & Krause, D. C. (1987).** Nucleotide sequence of the *Rickettsia prowazekii* citrate synthase gene. *J Bacteriol* **169**, 3564–3572.

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